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1653#18
3/18/02

Practitioner's Docket No. U012104-2 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: RAMA **MUKHERJEE**, ET AL.
Serial No.: 09/248,382 Group No.: 1653
Filed: FEBRUARY 10, 1999 Examiner: F. MOEZIE
For: NOVEL PEPTIDE ANALOGS FOR THE TREATMENT OF CANCER

Assistant Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL OF CERTIFIED COPY

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: INDIA

Application Number: 343/DEL/98

Filing Date: 11 FEBRUARY 1998

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Date: February 4, 2002

Signature

Janet I. Cord
(*type or print name of person certifying*)

SIGNATURE OF PRACTITIONER

Reg. No. 33,778

Janet I. Cord
(type or print name of practitioner)

Tel. No.: (212) 708-1935

P.O. Address

Customer No.: 00140

c/o Ladas & Parry
26 West 61st Street
New York, N.Y. 10023

NOTE: *"The claim to priority need be in no special form and may be made by the attorney or agent, if the foreign application is referred to in the oath or declaration, as required by § 1.63."* 37 C.F.R. 1.55(a).

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W-5, WEST PATEL NAGAR,
NEW DELHI-110 008.

*I the undersigned being an officer duly authorised in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the Application and Complete Specification filed in connection with Application for Patent No.**343/Del/98** dated **11th February 1998.***

*Witness my hand this **26th** day of **September** 2001.*

(H.C. BAKSHI)
Deputy Controller of Patents & Designs.

0343 DEL 98

11 FEB 1998

FORM 1A
THE PATENTS ACT, 1970
APPLICATION FOR PATENT

By the assignee or Legal Representative of the True and First Inventor.
[See Section 7]

We, NATIONAL INSTITUTE OF IMMUNOLOGY, a Society registered under the Indian Society's Registration Act XXI, of Aruna Asaf Ali Marg, New Delhi-110 067, India and Dabur Research Foundation, of 22, Site IV Sahibabad, Ghaziabad-201 010, (U.P).

hereby declare:-

i] that we are in possession of an invention for
"NOVEL PEPTIDE ANALOGS FOR THE TREATMENT OF CANCER"

ii] that we claim to be the assignees of RAMA MUKHERJEE, an Indian citizen of National Institute of Immunology, of Aruna Asaf Ali Marg, New Delhi-110 067 and MANU JAGGI, SUDHANAND PRASAD AND ANAND C. BURMAN, all Indian citizens, of Dabur Research Foundation, 22, Site IV Sahibabad, Ghaziabad-201 010, (U.P),

who claim and are believed to be the true and first inventors thereof;

iii] that the complete specification filed with this application and any amended specification which may hereafter be filed in this behalf will be true of the invention to which this application relates;

DUPLICATE

iv] that we believe that we are entitled to a patent for the said invention having regard to the provisions of The Patents Act, 1970;

v] that to the best of our knowledge, information and belief, the facts and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

We, request that a patent may be granted to us for the said invention.

We request that all notices, requisitions, and communications relating to this application may be sent to REMFRY & SAGAR, Attorneys-at-Law, Remfry House, 8, Nangal Raya Business Centre, New Delhi-110 046.

Dated this 11th day of February, 1998

OF REMFRY & SAGAR
ATTORNEY FOR THE APPLICANTS

TO
THE CONTROLLER OF PATENTS
THE PATENT OFFICE
DELHI

343/Del/98

THE PATENTS ACT 1970

COMPETE SPECIFICATION

(Section 10)

NOVEL PEPTIDE ANALOGS FOR THE TREATMENT OF CANCER

National Institute of Immunology and Dabur Research Foundation, a Society registed under the Indian Society's Registration Act XXI, of Aruna Asaf Ali Marg, New Delhi - 110067, India and of 22 Site IV Shaibabad, Gaziabad - 2001 010, UP, India, respectively.

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed:-

NOVEL PEPTIDE ANALOGS FOR THE TREATMENT OF CANCER

FIELD OF INVENTION

This invention relates to design, synthesis and anticancer activity of novel peptide analogs of vasoactive intestinal peptide, somatostatin, bombesin and substance P. Neuropeptide analogs are increasingly implicated in treatment of cancers.

BACKGROUND OF THE INVENTION

The neuropeptides vasoactive intestinal peptide and bombesin exert physiological effects by binding to specific receptors present on cells in the gastrointestinal tract and central nervous system. Bombesin is an amphibian peptide that has a structure closely related to that of several mammalian peptides, including Gastrin Releasing Peptide (GRP) and Neuromedins I and C. It was discovered in 1970 as a potent smooth muscle contracting agent of nonmammalian origin first isolated from amphibian skin (Erspamer et al., J Pharm. Pharmacol. 22:275 (1970). Bombesin, GRP and related peptides exert their in vivo effects by binding to specific receptors present on cells of the gastrointestinal tract, the central nervous system and tumors. The invention relates to novel bombesin analogs which act as antagonist of bombesin or related peptides such as gastrin releasing peptide. By blocking the binding of bombesin-like peptides to their receptors, these antagonists block the physiological effects of these peptides and inhibit the growth of tumor cells that respond to growth-promoting action of bombesin. Thus these antagonists have therapeutic use in the treatment or prevention of cancer and in controlling physiological effects in gastrointestinal disorders and in modulating responses of the central nervous system

The invention also relates to novel vasoactive intestinal peptide analogs which act as antagonists of VIP by blocking the binding of VIP to its cognate receptors. By blocking the binding of VIP to its receptor these analogs inhibit the growth of tumor cells that respond to the

growth-promoting action of VIP. VIP is a 28-amino-acid neuropeptide, which has been implicated as a major growth promoting factor during embryonic growth. In cancer cells, previous studies have implied that VIP can serve as an autocrine growth factor in lung tumors (Gozes et al. Biomed Res. 13 (Suppl. 2), 37. (1992)).

In our US Application 08/727,679 we have described the role of neuropeptides in cancer. High affinity and moderate affinity receptors for vasoactive intestinal peptide and somatostatin, high affinity receptors for bombesin and moderate affinity receptors for substance P were demonstrated on human colon adenocarcinoma cells. It was further demonstrated that peptide analogs to the above neuropeptides could actively and selectively induce cell death in the cancer cells. A formulation of peptide combination termed MuJ-7 has also been described which causes tumor regression in xenotransplanted nude mice. The individual constituent peptides of MuJ-7 were demonstrated to have anticancer activity.

Our further research work has led to the designing and testing of novel structural analogs of VIP receptor binding inhibitor and bombesin antagonist (both constituent peptides of MuJ-7 formulation) which have been designed to render conformational constraints and higher stability to the peptides while maintaining their anticancer activity. Substitutions and / or eliminations have been incorporated into VIP_2 and BOM_1 sequences keeping in mind not to alter amino acids known to offer conformational constraint and stability to the peptide. In order to introduce conformational constraints, unusual amino acids such as cyclic and acyclic dialkylated Glycines have been incorporated into the peptide backbone. Examples of such amino acids are Aib, Me, Leu, Di-ethylglycine and its higher homologs, 1-amino cycloalkane carboxylic acids.

SUMMARY OF THE INVENTION

The invention provides novel peptides for the treatment of cancer. The VIP receptor binding inhibitor (Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for vasoactive intestinal peptide. In our present studies we have designed new analogs of this sequence in order to introduce conformational constraints and resist enzymatic degradation. The peptides thus synthesised were

DT-11	Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH
DT-12	D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH
DT-13	Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH
DT-14	Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH
DT-15	Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH
DT-16	D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH
DT-18	Aib-Met-Tyr-Pro-Thr-Tyr-Dxg-Lys-OH
DT-19	D-Leu-Met-Tyr-Pro-Thr-Tyr-Dxg-Lys-OH

where Dxg represents Cyclic and acyclic dialkylated Glycines e.g., Diethylglycine and its higher homologs, 1-amino cycloalkane carboxylic acids and Aib represents alpha-Amino isobutyric acid.

The bombesin antagonist (D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for bombesin. In our present studies we have designed new analogs of this sequence in order to introduce conformational constraints and resist enzymatic degradation. The peptides thus synthesised are

DT21	D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NH_2

DT22 D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2

DT-23 D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2

DT-24 D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2

DT-25 D-Phe-Gln-Trp-Ala-Val-Gly-His-Ile-NH_2

DT-26 D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2

DT-27 D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2

where Aib represents alpha-Amino isobutyric acid.

The Substance P analog (D-Arg-Pro-Lys-Pro-Ala-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Substance P. In our present studies we have designed new analogs of this sequence in order to introduce conformational constraints and resist enzymatic degradation. The peptides thus synthesised are

DT-31 Aib-Met-Gln-Trp-Phe-Aib-NH_2

DT-32 Dxg-Met-Gln-Trp-Phe-Aib-NH^2

DT-33 D-Leu-Met-Gln-Trp-Phe-Aib-NH_2

DT-34 D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2

DT-35 Arg-Pro-Aib-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2

where Dxg represents Cyclic and acyclic dialkylated Glycines e.g., Diethylglycine and its higher homologs, 1-amino cycloalkane carboxylic acids and Aib represents alpha-Amino isobutyric acid.

The Somatostatin analog Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-D-Cys (disulfide bridges: 3-14) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Somatostatin. In our present studies we have

designed new analogs of this sequence in order to introduce conformational constraints and resist enzymatic degradation. The peptides thus synthesised are

DT-61 Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-lys-Thr-Phe-Thr-D-Ser-Cys (3-14 Disulphide bond)

where Aib represents alpha-Amino isobutyric acid.

The Somatostatin analog (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Somatostatin. In our present studies we have designed new analogs of this sequence in order to introduce conformational constraints and resist enzymatic degradation. The peptide thus synthesised is

DT-71 D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2

where Aib represents alpha-Amino isobutyric acid.

EXAMPLE 1

The cytotoxic activity of the peptides synthesised was tested on eight human tumor cell lines namely HT-29, SW620, PTC (all colon), PA-1 (ovary), A549 (lung), HBL100 (breast), MOLT-4 (leukemia) and DU145 (prostate). The tumor cells were collected at exponential growth phase and resuspended in medium (1.5 x 10^6 cells/ml in RPMI 1640 containing 10% FBS). 150µl of medium was added to the wells of a 96-well tissue culture plate (Nunc, Denmark) followed by 30µl of cell suspension. The plate was left in incubator (37^0C, 5% CO_2) overnight. 20µl of the peptide (10^{-7} to 10^{-10}M concentration) was added to marked wells of the 96-well plate. Each concentration was plated in triplicates. 20µl of medium alone was added to control wells while wells without cells served as blanks. A total volume of 200µl was ensured in each well and plate was left in incubator (37^0C, 5% CO_2). After 72 hours of

incubation an MTT assay was performed and percentage cytotoxicity was calculated with respect to control cells. The following Tables I to V show the results obtained.

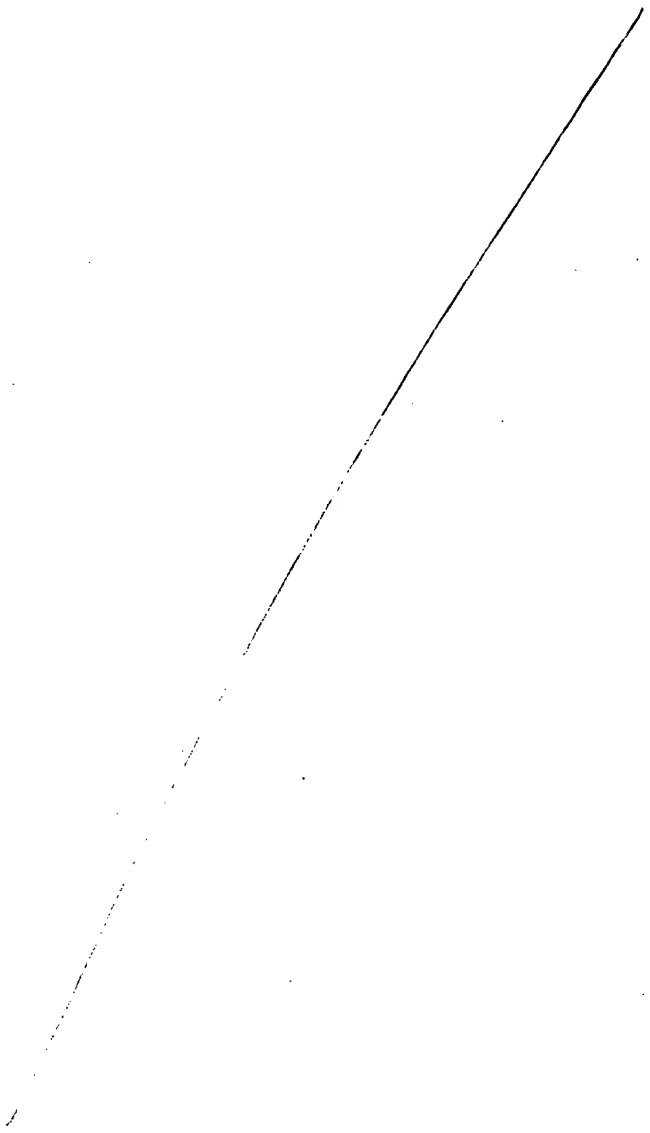

TABLE - I

Percent cytotoxicity of DT-1 analogs on various tumor cell lines

Cell line	DT11	DT12	DT13	DT14	DT15	DT16	DT18	DT19
PA-1	32	26	23	14	25	22	31	34
PTC	16	24	20	0	23	18	14	10
MOLT-4	30	42	39	30	33	31	36	23
HBL100	24	19	27	19	18	2	24	27
A549	29	36	32	42	37	33	27	25
SW620	13	0	18	12	28	22	26	33
HT29	0	7	6	6	11	20	38	30
DU145	7	19	1	5	10	0	18	24

TABLE - II

Percent cytotoxicity of DT-2 analogs on various tumor cell lines

Cell line	DT21	DT22	DT23	DT24	DT25	DT26	DT27
PA-1	23	24	16	33	21	24	26
PTC	18	12	15	23	9	25	12
MOLT-4	25	17	29	8	23	20	13
HBL100	27	14	33	32	25	6	16
A549	16	22	23	30	25	13	18
SW620	25	33	34	38	31	38	32
HT29	24	35	43	44	40	27	28
DU145	10	22	25	32	33	24	0

TABLE III

Percent cytotoxicity of DT-3 analogs on various tumor cell lines

Cell line	DT31	DT32	DT33	DT34	DT35
PA-1	24	ND	ND	ND	ND
PTC	21	32	23	16	18
MOLT-4	30	32	37	26	20
HBL100	15	16	15	21	15
A549	23	19	21	24	20
SW620	14	ND	ND	ND	ND
HT29	30	13	18	9	26
DU145	25	ND	ND	ND	ND

TABLE IV

Percent cytotoxicity of DT-6 analogs on various tumor cell lines

Cell line	DT61
PA-1	45
PTC	22
MOLT-4	34
HBL100	26
A549	28
SW620	26
HT29	35
DU145	29

TAVLE V

Percent cytotoxicity of DT-7 analogs on various tumor cell lines

Cell line	DT71
PA-1	ND
PTC	19
MOLT-4	37
HBL100	23
A549	18
SW620	ND
HT29	19
DU145	ND

EXAMPLE 2

A 0.5 mL of 2000 ppm of the VIP_{2a} was mixed with 1.0 ml freshly prepared liver homogenate to obtain a concentration of 1000 ppm. Sample preparations was incubated at 37° C and after time intervals of 0, 2, 5, 10, 20 and 30 minutes, 200µl of the preparation was aliquot and precipitated with equal volumes of acetonitrile. Incase of BOM_1 analogs, the sample preparations with a final concentration of 1000 ppm were incubated at 37° C and after time intervals of 0, 15, 30, 60, 90, 120 and 150 minutes, 200µl of the preparation was aliquot and precipitated with equal volumes of acetonitrile. The precipitate was pelleted by centrifugation at 10,000 g for 5 min and supernatant analyzed by HPLC. The percentage increase in the half-life of DT1 and DT2 analogs with reference to DT1 and DT2, respectively, as estimated by the mouse liver homogenate study, is shown in Tables VI and VII respectively.

Preparation Of Mouse Liver Homogenate

1 Healthy Balb/c mouse was sacrificed and dissected to expose liver.

2 The pulmonary artery was severed to drain blood and cold saline was perfused through portal vein till liver becomes pale white.

3 The liver was excised ,minced and passed through 60# sieve.

4 1.15% w/v KCl -0.01M phosphate buffer (pH 7.4) was added to make 20% w/v homogenate that was centrifuged at 4500 g for 15 min.

5 The supernatant was recovered and further centrifuged at 10,000 g to clarify the homogenate.

TABLE VI

Half life of DT-1 analogs with reference to
DT-1 as determined by the mouse liver homogenate study

Peptide	Half-life (minutes)
DT-1	4.9
DT-11	15.4
DT-12	18.1

TABLE VII

Half life of DT-2 analogs with reference to
DT-2 as determined by the mouse liver homogenate study

Peptide	Half-life (minutes)
DT-2	12.6
DT-22	15.06
DT-23	57.7
DT-24	38.5
DT-26	114.5
DT-27	292.5

WE CLAIM:-

1. A Peptide having the general formula Leu^{1}-Met^{2}-Tyr^{3}-Pro^{4}-Thr^{5}-Tyr^{6}-Leu^{7}-Lys^{8}.

2. A peptide according to claim 1, wherein any one or more of the amino acids at positions 1 to 8 is replaced by Dxg wherein Dxg represents cyclic and acyclic dialkylated Glycines such as Aib, Di-ethylglycine and its higher homologs and 1-amino cycloalkane carboxylic acids.

3. A peptide according to claim 1 or 2, wherein the peptide is used for the treatment of cancer of colon, breast, ovary, lung, prostate or leukemias.

4. A peptide having the general formula D-Phe^{1}-Gln^{2}-Trp^{3}-Ala^{4}-Val^{5}-Gly^{6}-His^{7}-Leu^{8}-NH_2.

5. A peptide according to claim 4, wherein any one or more of the amino acids at positions 1 to 8 is replaced by Dxg wherein Dxg represents cyclic and acyclic dialkylated Glycines such as Aib, Di-ethylglycine and its higher homologs and 1-amino cycloalkane carboxylic acids.

6. A peptide according to claim 4 or 5, wherein leucine is replaced with isoleucine.

7. A peptide according to claim 4, 5 or 6, wherein tryptophan is replaced with D- tryptophan.

8. A peptide according to any one of claims 4 to 7, wherein the peptide is used for the treatment of cancer of colon, breast, ovary, lung, prostate or leukemias.

9. A peptide having the general formula D-Arg^{1}-Pro^{2}-Lys^{3}-Pro^{4}-D-Phe^{5}-Gln^{6}-D-Trp^{7}-Phe^{8}-D-Trp^{9}-Leu^{10}-Leu^{11}-NH_2.

10. A peptide according to claim 9, wherein the length of the peptide is 5 to 11 amino acids and amino acids at positions 1 to 11 is replaced by Dxg and/or Aib at any one or more given positions, wherein Dxg represents cyclic and acyclic dialkylated Glycines such as Aib, Di-ethylglycine and its higher homologs and 1-amino cycloalkane carboxylic acids.

11. A peptide according to claim 9 or 10, wherein the peptide is used for the treatment of cancer of colon, breast, ovary, lung, prostate or leukemias.

12. A peptide having the general formula Ala^{1}-Gly^{2}-Cys^{3}-Lys^{4}-Asn^{5}-Phe^{6}-Phe^{7}-D-Trp^{8}-Lys^{9}-Thr-^{10}-Phe^{11}-Thr^{12}-Ser^{13}-D-Cys^{14} (dislfide 3-14 bridge).

13. A peptide according to claim 12, wherein amino acids at positions 1 to 14 may be replaced by Dxg and/or Aib at any one or more given positions, wherein Dxg represents cyclic and acyclic dialkylated Glycines such as Aib, Di-ethylglycine and its higher homologs and 1-amino cycloalkane carboxylic acids.

14. A peptide according to claim 12 or 13, wherein the peptide is used for the treatment of cancer of colon, breast, ovary, lung, prostate or leukemias.

15. A peptide having the general formula D-Phe^{1}-Cys^{2}-Tyr^{3}-D-Trp^{4}-Orn^{5}-Thr^{6}-Pen^{7}-Thr^{8}-NH_2 (Disulfide 2-7 bridge).

16. A peptide according to claim 15, wherein amino acids at positions 1 to 8 is replaced by Dxg and/or Aib at any one or more given positions, wherein Dxg represents cyclic and acyclic dialkylated Glycines such as Di-ethylglycine and its higher homologs and 1-amino cycloalkane carboxylic acids.

17. A peptide according to claim 15 or 16, wherein the peptide is used for the treatment of cancer of colon, breast, ovary, lung, prostate or leukemias.

18. Peptide analogs for the treatment of cancer substantially as hereinbefore described and illustrated with reference to the foregoing Examples.

Dated this 11th day of February 1999.

[K. T. Jose]
Of REMFRY & SAGAR
ATTORNEY FOR THE APPLICANTS